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**Diagnosis and monitoring for light chain only and oligosecretory myeloma
using serum free light chain tests**

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Running head: Serum free light chains in myeloma

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Summary

This study aims to guide integration of serum FLC (sFLC) tests into clinical practice, including a new rapid test (Seralite®). Blood and urine analysis from 5573 newly diagnosed myeloma patients provided 576 light chain only (LCO) and 60 non-secretory (NS) cases. Serum was tested by Freelite® and Seralite® at diagnosis, maximum response and relapse. 20% of LCO patients had urine FLC levels below that recommended for measuring response but >97% of these had adequate sFLC levels (oligosecretory). The recommended Freelite® FLC >100mg/L for measuring response was confirmed and equivalent Seralite® FLC difference (dFLC) >20mg/L identified. By both methods, ≥ 38% of NS patients had measurable disease (oligosecretory). Higher sFLC levels were observed on Freelite® at all time points. However, good clinical concordance was observed at diagnosis and in response to therapy. Achieving ≥ VGPR, according to either sFLC method, was associated with better patient survival. Relapse was identified using a Freelite® sFLC increase >200mg/L and found 100% concordance with a corresponding Seralite® dFLC increase >30mg/L. Both Freelite® and Seralite® sensitively diagnose and monitor LCO/oligosecretory myeloma. Rapid testing by Seralite® could fast-track FLC screening/monitoring. Response by sFLC assessment was prognostic for survival and demonstrates the clinical value of routine sFLC testing.

Key Words: free light chains, multiple myeloma, serum, non-secretory, quantitation, survival

Introduction

The measurement of monoclonal protein (M-protein), both whole immunoglobulin and free light chains (FLC), in blood and urine is fundamental to the diagnosis and monitoring of all plasma cell dyscrasias. For most patients the serum whole M-protein remains the main focus of laboratory attention, however, in up to a fifth of all myeloma patients there is no detectable intact M-protein (light chain only [LCO] patients) and so FLC detection and quantitation is essential (Drayson, *et al* 2006). FLC measurement was first established in urine (uFLC) and quantitation in serum (sFLC) was not available until 2001.

The first commercially available sFLC assay (Freelite®) utilises sheep polyclonal antibodies highly specific for the epitopes exposed on LC that are not bound to heavy chain (Bradwell, *et al* 2001). The greater sensitivity of measuring FLC levels in serum versus urine was shown in the detection of abnormal sFLC levels in 19/28 non-secretory (NS) myeloma patients with undetectable FLC in serum and urine by immunofixation electrophoresis (IFE); these 19 myeloma patients could be redefined from NS to oligosecretory myeloma (Drayson, *et al* 2001). The sFLC test reliably identified 224 LCO patients at diagnosis and the greater sensitivity was seen again at maximum response, where sFLC levels remained abnormal in two thirds of patients with no FLC detectable in urine (Bradwell, *et al* 2003). SFLC testing has subsequently been incorporated into International Myeloma Working Group Guidelines for diagnosis and management of all plasma cell dyscrasias (Dispenzieri, *et al* 2009, Durie, *et al* 2006). However, these guidelines still recommend use of uFLC for measurement of response to therapy if available because there is insufficient published data to advocate replacement with sFLC testing, although the evidence is increasing (Dejoie, *et al* 2016a, Dejoie, *et al* 2016b). Further, it is not clear how sFLC levels at diagnosis and in response to therapy compare in patients with measurable disease in urine or those with low or undetectable uFLC levels.

Current International Myeloma Working Group guidelines are based on the Freelite® assay (Dispenzieri, *et al* 2009, Durie, *et al* 2006). Freelite® and N Latex, another FLC assay that has become available more recently, require nephelometric or turbidimetric analysers. These assays are not available in many biochemistry departments requiring samples to be sent away to

specialised laboratories. Processing samples at external sites can lead to slow turn-around times, potentially delaying clinical interventions while physicians await their patient's test results. A portable sFLC test has been developed (Seralite®, Abingdon Health Ltd, UK) that quantitates serum κ and λ FLC levels simultaneously in 10 minutes, with demonstrated clinical specificity (Campbell, *et al* 2017). This rapid lateral-flow device could aid in the acceleration of myeloma diagnosis and facilitate prompt feedback on patient responses to anti-myeloma therapy.

As is found for measurement of intact M-proteins by different methods, each FLC assay often quantitates absolute monoclonal/involved FLC (iFLC) differently in individual patients (Te Velthuis, *et al* 2016). To enable further incorporation of sFLC measurement into clinical practice, and the utilisation of new technologies, there needs to be extensive assessment of clinical concordance between these methods of monitoring disease activity. This requires comparative evaluation of FLC levels at diagnosis, response to therapy and relapse. It is important to evaluate how the recommended guideline thresholds for Freelite® perform in clinical samples and establish appropriate thresholds for new tests, such as Seralite®. Further, to verify the clinical utility of sFLC assessment, it is imperative to understand how sFLC measurements on both these assays relate to patient outcomes.

The present study is based on central laboratory analysis of 5573 newly diagnosed myeloma patients and aims to (I) characterise sFLCs in LCO and NS myeloma at diagnosis, (II) increase understanding of sFLCs in relation to urine, (III) compare sFLC quantitation between the Freelite® assay and the Seralite® test at diagnosis and response (IV) assess the threshold sFLC levels that are sufficient for monitoring response to therapy and for defining relapse by both Freelite® and Seralite® and (V) evaluate sFLC response assessment by Freelite® and Seralite® in relation to survival.

Methods

Patients

Patients included in the present analyses were enrolled in multi-centre, phase III national myeloma trials for newly diagnosed patients in the United Kingdom between 2003 and 2015: either the

Medical Research Council/National Cancer Research Institute Myeloma IX trial (ISRCTN68454111, n = 1693) or the ongoing Cancer Research UK Myeloma XI trial (ISRCTN49407852, n = 3894), up to an enrolment date of July 2015. We reviewed central laboratory tests and evaluated individuals classified as LCO or NS with paired serum and urine data available at disease presentation.

All patient serum and urine samples were assessed by IFE (Sebia, France) to determine if monoclonal FLCs were present. Subsequently, FLC data from Freelite® were retrospectively evaluated on 576 patients diagnosed with LCO myeloma and 60 with NS myeloma. Where archived presentation sera were available in adequate volume, samples underwent further FLC analyses using the lateral flow device Seralite® (n = 325) for comparison with Freelite®. A cohort of LCO myeloma patients with follow-up sera samples available were identified and analysed at maximum response (n = 163) and relapse (n = 40) using Seralite® to enable comparison with documented Freelite® results.

Patient characterisation at diagnosis and assessment of response

Light chain only patients were defined as patients positive for monoclonal FLCs on urine IFE and negative for intact monoclonal protein on serum IFE. These patients were then divided into those patients who did/did not meet the urine FLC (uFLC) guideline threshold (GLT) required to measure response to therapy. The uFLC GLT applied was 200 mg/g creatinine. International criteria GLT are based upon 200 mg/24h, however, it has been shown previously that 200 mg/g creatinine equates to this level and that spot urine protein to creatinine ratios are reliable and easier to undertake than measurements in 24 hour urine collections (Le Mouel, *et al* 2015, Matar, *et al* 2012). Those with uFLC < 200 mg/g creatinine were inspected for serum FLCs to determine if patients could be monitored. A sFLC GLT of 100 mg/L on Freelite® was applied to determine if patients had sufficient sFLC levels to reliably measure response to therapy (Durie, *et al* 2006). Patients meeting this threshold were subsequently defined as oligosecretory. NS patients were negative for monoclonal protein (intact immunoglobulin and FLC) on both serum and urine IFE and with clinical and bone marrow data confirming diagnosis for inclusion in the myeloma trial. The sFLC GLT of 100 mg/L was applied to NS patients (able to be diagnosed via an abnormal $\kappa:\lambda$ ratio)

to identify those with oligosecretory disease who would have been suitable for monitoring with sFLCs. Patient classification is described in full Figure 1.

Patient's maximum response to therapy was categorised using conventional international response criteria (Durie, *et al* 2006, Rajkumar, *et al* 2011): stable disease (SD) if difference in kappa and lambda FLC levels (dFLC) reduced by < 50%; a partial response (PR) if dFLC reduced by \geq 50%; a very good partial response (VGPR) if dFLC reduced by \geq 90%. Complete response (CR) was defined according to conventional criteria – a normal $\kappa:\lambda$ ratio, and also by normalisation of dFLC, according to Freelite® or Seralite®. Normalisation of the dFLC was explored as an alternative to the $\kappa:\lambda$ ratio as anti-myeloma therapy often results in immunosuppression of the uninvolved FLC to levels below the sensitivity of the assays making the FLC ratio unreliable. Other response categories already employ the dFLC and this parameters may be more relevant for clinical outcomes and thus more suitable for assignment of CR vs the $\kappa:\lambda$ ratio. International guidelines advise a dFLC increase of > 25% and an absolute increase > 100 mg/L by Freelite® to indicate progressive disease in patients with unmeasurable FLC in urine (Durie, *et al* 2006). In the absence of clinical symptoms, an iFLC increase > 200 mg/L is recommended for defining relapse requiring treatment (Rajkumar, *et al* 2011). In this study, patients with relapse requiring treatment (iFLC > 200 mg/L Freelite®) were identified in patients with follow-up serum samples available. All patients also had a dFLC increase from maximum response of greater than 25%.

Free light chain quantitation

All patient serum samples underwent central laboratory analysis for FLCs using Freelite®. Where stored serum was available, samples were measured using Seralite®. Seralite® is a recently developed portable lateral-flow test designed for near-patient testing that utilises anti-FLC mouse monoclonal antibodies (mAbs) (Abingdon Health Ltd, York, UK) enabling rapid simultaneous quantification of κ and λ FLCs (described in full elsewhere) (Campbell, *et al* 2013, Campbell, *et al* 2017). Seralite® has been shown to have good precision in samples with varying levels of FLC when measured both across the day (CV \leq 9.6%) and between days (CV% \leq 7.1%). These low levels of imprecision are comparable with other sFLC quantitation methods, with CVs of < 10% reported for Freelite and < 6% for N Latex® (Bradwell, *et al* 2001, te Velthuis, *et al* 2011). From

2002 to 2008, routine assessment of FLCs in urine at disease presentation was conducted using radial immunodiffusion (The Binding Site Ltd, Birmingham, UK). Post-2008, quantification of κ and λ FLCs in urine was replaced by the Luminex mAb assay, which employs the same anti-FLC mAbs as used in Seralite® in a multi-plex bead array clinically validated for urine (Campbell, *et al* 2013). The Luminex mAb assay clinical specificity was evaluated using 13090 urine specimens sent for central laboratory analysis for routine assessment of FLCs. The assay identified FLCs in all 4175 samples that contained monoclonal FLC detectable by urine IFE, the gold standard for identifying FLC in urine. Urine FLC levels were adjusted for creatinine, which was measured using a Roche Hitachi Modular (Basel, Switzerland).

Data analyses

Descriptive statistics were used to evaluate NS myeloma patients and compare sFLC results between methods. In the NS patients, a receiver operating characteristic (ROC) curve was generated to assess if dFLC on Seralite® could discriminate patients with > 100 mg/L on Freelite® and the best dFLC cut-off was determined. LCO patients were divided into two groups: LCO and OS as described above; these groups were retained throughout data analyses. Mann Whitney U-tests were used to compare between LCO and OS myeloma groups. For evaluation of paired Seralite® and Freelite® data, Wilcoxon signed-rank tests were employed. Chi-square was used for analyses of categorical variables. Survival outcomes were analysed between patients who achieved a VGPR or CR and those who achieved below a VGPR at max response. Survival curves were constructed using the method of Kaplan & Meier and the log-rank test was used to assess differences between sFLC response, by Freelite® or Seralite®. Hazard ratios and 95% confidence intervals were estimated using Cox proportional hazards regression. In patients with a VGPR, survival curves were also constructed in relation to normalisation of the dFLC vs abnormal dFLC and normalisation of the κ : λ ratio vs abnormal ratio. At the date of download (25th July 2016) median follow-up in NCRI Myeloma XI was 35.7 months. Overall survival (OS) was defined as the time from the date of initial randomisation to the trial to the date of death from any cause or last follow-up. If a participant were still alive at the time of analysis or lost to follow-up before death was documented, they were censored at the last date known alive. Progression-free survival

(PFS) was defined as the time from the date of initial randomisation to the trial to the date of progression or death from any cause. Participants who did not progress were censored at the last date they were known to be alive and progression-free.

Results

Light chain only myeloma patients at disease presentation

A total of 576 patients were classified as LCO myeloma because they did not have an intact M-protein on serum IFE and did have monoclonal FLC detected by urine IFE. Of these patients, 81% also had a monoclonal FLC detectable in serum by IFE. Notably, regardless of uFLC levels, the serum $\kappa:\lambda$ ratio was abnormal in all patients thus able to sensitively diagnose LCO myeloma independently from urine results. Figure 1 describes patient characteristics in relation to GLTs for all LCO patients ($n = 576$) and then LCO patients with matched Freelite® and Seralite® data ($n = 325$). For all patients, 460 (80%) had uFLC levels \geq GLT sufficient to monitor response; the majority of these patients (454, 99%) also had sFLC levels \geq GLT and thus would also qualify to be monitored via serum. For the remaining 116 with uFLC $<$ GLT, 113 (97%) had sFLC levels \geq GLT sufficient to measure response to therapy - leading to re-classification as OS patients. The other 3 patients had unmeasurable disease by both uFLC and sFLC. For the paired Freelite® and Seralite® data, 253 (78%) had uFLC levels \geq GLT sufficient to monitor response. The remaining 72 patients all had sFLC levels $>$ GLT leading to re-classification as OS patients. Descriptive statistics for LCO and OS patients are displayed in Supplementary Table I. LCO patients showed significantly higher serum levels of iFLC compared to oligosecretory patients for both FLC methods ($p < .001$).

Figure 2 displays serum iFLC for all patients on Freelite® and the sub-cohort of these patients who had matched Freelite® and Seralite® data. For both LCO and OS patient groups, a significant difference between methods was found for serum iFLC levels, where Freelite® levels were significantly higher compared to Seralite® ($p < .001$). Freelite iFLC concentrations were at least 5 times higher than results generated by Seralite® (Supplementary Table I). Values for the dFLC were almost identical to the iFLC, due to suppression of the alternate FLC.

In Figure 3, FLC κ : λ ratio results are compared between Freelite® and Seralite®. All LCO patients displayed an abnormal κ : λ ratio on both Freelite® and Seralite® (100% concordance). For OS patients, abnormal ratios were observed for all 72 patients on Freelite® and 69/72 on Seralite® (95% concordance). In the 3 OS patients with normal serum FLC ratios by Seralite®, serum was negative for monoclonal FLC by IFE but the anti-FLC mAbs used in the Seralite® test did detect FLCs in the urine of these patients (measured on the Luminex platform). This may reflect renal tubular dysfunction allowing appearance of monoclonal FLC in urine before the serum FLC ratio becomes substantially abnormal.

Nonsecretory myeloma at disease presentation

At diagnosis, 60 patients were identified with both serum and urine samples negative for both monoclonal intact immunoglobulin and FLC on IFE. Full sFLC results for individual patients are described in Supplementary Table II. Elevated κ or λ FLCs and abnormal ratios were detectable by at least one of the FLC assays in 31 (52%) patients. In these 31 patients, 23 (74%) presented with the iFLC \geq GLT on Freelite® deemed to be sufficient for reliable measurement of response (100 mg/L). Discordance between Freelite® and Seralite® for κ : λ ratios occurred in 6 of these 31 patients; 1 patient had a normal κ : λ ratio on Freelite® and 5 patients had a normal κ : λ ratio on Seralite®. In patients where Seralite® did not detect an abnormal ratio, 4/5 had Freelite® iFLC levels $<$ GLT and thus would not be suitable for monitoring using sFLCs. In the 31 patients with elevated FLCs and abnormal ratios, the frequency of κ vs λ myelomas was 27:4 in contrast to LCO patients, where a ratio of 1.5:1 for κ to λ diagnosis occurred ($X^2 = 9.38$, $p < .05$). Suppression of both kappa and lambda FLC levels below the normal range was seen in a further 30% of patients; in two thirds of these cases the κ : λ ratio was normal and in the other third considered unreliable because the assays were measuring both the kappa and lambda levels at the lower limits of their sensitivity. The remaining 18.3% of patients had normal/borderline κ or λ levels or normal ratios.

Defining measurable disease at presentation

SFLC results from patients with NS myeloma (presenting with abnormal κ : λ ratios and elevated FLCs) were reviewed in relation to Seralite® to assign an appropriate GLT for measurable disease at diagnosis. As quantitation differed between the two methods, the dFLC rather than iFLC was

employed for Seralite®. Receiver operating characteristic (ROC curve) analysis showed that the Seralite® dFLC was able to accurately identify patients with > 100 mg/L on Freelite®, AUC = .85 (95% CI .63–.1, $p < .05$). The best dFLC cut-off was 21.2 mg/L with a sensitivity of 92% and specificity of 75%. For user ease of use in practice, a dFLC of 20 mg/L on Seralite® was selected as the GLT to discriminate measurable disease at diagnosis. This level was twice the level of the upper limit of the dFLC normal range observed in healthy donors. Overall in NS patients, 23/60 (38%) patients tested by Freelite® had an abnormal ratio and iFLC >100mg/L; 14/35 (40%) patients tested by Seralite® had an abnormal ratio and dFLC >20mg/L. Patients with LCO myeloma with available Seralite® results were re-evaluated at diagnosis with this Seralite® GLT of >20mg/L dFLC. For OS patients, unsuitable for urine monitoring, 65/72 (90%) had an abnormal FLC ratio and dFLC > 20 mg/L. For LCO patients, 251/253 (99%) would also be above this dFLC threshold (for all LCO patients 316/325 (97%) dFLC > 20 mg/L).

Follow-up of light chain only patients at maximum response

163 patients with measurable disease at diagnosis (according to both Freelite® and Seralite® GLTs) had serum samples available at max response (Table I). At max response, the dFLC returned negative values in some patients, reflecting the involved FLC returning to normal or below normal levels with therapy (below the uninvolved FLC). For both methods, there was a significant difference in therapy responses between LCO and OS patients ($X^2 = 7.59$, $p < .05$ Freelite®; $X^2 = 11.96$, $p < .01$ Seralite®). Proportionately more OS patients obtained a CR (both by normalised ratio and normalised dFLC) where more LCO patients achieved a VGPR. More patients achieved a normalised dFLC compared to normalised $\kappa:\lambda$ ratio for both LCO and OS patients, according to both methods.

The absolute levels of dFLC were significantly higher on Freelite® compared with Seralite® for both patient groups. However, the median percentage reduction in dFLC from presentation to max response was the same between the two methods. Within the LCO group, a difference was observed in response categories measured by the two FLC methods, $X^2 = 8.74$, $p < .05$ (Table I). More patients obtained a CR when $\kappa:\lambda$ ratios were determined by Seralite® compared to Freelite®, and alternatively, more patients were categorised with a VGPR on Freelite® compared to

Seralite®. However, there was little difference between the methods for good response rates as 88% achieved a VGPR or better on Freelite® compared with 83% on Seralite®. In the OS patients, there was no statistically significant difference in responses between methods.

Relationship between serum free light chain response assessment and survival outcomes

Survival outcomes were firstly explored in relation to sFLC response criteria assigned using Freelite® for all patients with measurable disease at diagnosis who had follow-up data available (n = 402). As illustrated in Figure 4, patients who achieved a VGPR or CR had significantly better PFS ($X^2 = 77.3$, $p < .0001$, HR 0.34 [0.27–0.44]) and OS ($X^2 = 41.6$, $p < .0001$, HR 0.37 [0.27–0.51]) compared to patients with PR or SD at max response. Patients with \geq VGPR had a 66% and 63% reduced risk of death/progression and death, respectively. There were no significant differences in PFS and OS between LCO, OS or NS patients. In patients who achieved \geq VGPR, there were no significant differences in survival between those who did/did not have a normalised $\kappa:\lambda$ ratio. However, a significant difference in PFS survival was observed on the basis of a normalised dFLC (Figure 4). Patients with a normalised dFLC had significantly better PFS compared to those who did not have a normal dFLC ($X^2 = 18.7$, $p < .0001$). Patients who had a normalised dFLC were at reduced risk of death/disease progression (HR 0.52 [0.39–0.71]). There was no significant difference in OS in relation to a normalised/abnormal dFLC.

Survival analyses were then repeated for the patients who had sFLC measured using Seralite® at max response. Patients who had \geq VGPR had significantly better PFS ($X^2 = 6.52$, $p < .05$, HR 0.54 [0.34–0.87]) and better OS ($X^2 = 3.60$, $p = .058$, HR 0.54 [0.28–1.03]) compared with those patients with a PR or SD. In patients who achieved \geq VGPR, normalisation of the dFLC was associated with significantly better PFS ($X^2 = 13.86$, $p < .001$) and OS ($X^2 = 10.01$, $p < .01$). Patients with a normal dFLC had a lower hazard of disease progression (HR 0.42 [0.26–0.67]) or death (HR 0.37 [0.19–0.70]) compared to patients with a non-normalised dFLC. Patients with a normalised ratio also had significantly better PFS compared to those with an abnormal ratio ($X^2 = 5.90$, $p < .05$, HR 0.57 [0.36–0.90]). There was no significant difference in OS between patients with/without a normalised $\kappa:\lambda$ ratio.

Follow-up of light chain only patients at relapse

SFLC parameters for patients at relapse analysed using Freelite® and Seralite® are shown in Table II. Only a small number of OS patients (n = 6) had serum available at this time point therefore the data combined with LCO patients. Similar to diagnosis and response, dFLC levels measured by Freelite® were higher compared with Seralite®. Percentages increases in dFLC from max response were also higher on Freelite®, but both methods demonstrated substantial percentage increases and were able to clearly identify a return in disease activity from remission. In these patients who presented with a relapsed iFLC absolute increase of > 200 mg/L on Freelite®, Seralite® iFLCs values of < 200 and also < 100 mg/L were observed. However, the increase in dFLC by Seralite® was consistently > 30 mg/L; this level corresponding to > 200 mg/L on Freelite® is an appropriate cut-off to define relapse using this FLC test. Figure 5 provides a summary of iFLC and dFLC levels on Freelite® and Seralite® at presentation, response and relapse. This figure illustrates that both methods of sFLC quantitation can effectively track LCO and OS patients over time. Seralite® generates lower absolute levels of FLCs relative to Freelite® at diagnosis and throughout monitoring; however, the two methods concord regarding the patient's disease activity relative to the previous time point. All patients at relapse presented with an abnormal $\kappa:\lambda$ FLC ratio, however, $\leq 30\%$ of these patients had a normalised ratio at max response; thus the ratio may only be useful in identifying active disease and not remission.

Discussion

FLC testing is essential in patients with LCO myeloma and sFLC assessment may be particularly valuable when urine FLC levels are low, urine samples are not received, and when serum IFE is negative. In the 576 LCO patients with newly diagnosed myeloma (all with uFLC detectable by IFE) 80% had uFLC levels sufficient to monitor response. Of these patients, almost all (99%) had FLC levels sufficient to measure response in serum as well. In patients with insufficient uFLC for monitoring, nearly all could be classed as OS with sFLCs able to measure response. Therefore, regardless of urine, most LCO patients would be suitable for monitoring with sFLCs. Out of this large patient sample, only 3 patients had unmeasurable disease by either serum or urine FLCs.

LCO patients presented with significantly higher levels of iFLC than OS patients. Although, importantly, the sFLC $\kappa:\lambda$ ratio was able to sensitively diagnose all patients independently of uFLC levels and patient grouping. In the present study uFLCs were determined using spot urine samples, rather than 24h specimens, and protein electrophoresis was not employed for quantitation. However, these data suggest that information derived from urine is not essential for the diagnosis of LCO myeloma, in line with current recommendations for screening monoclonal gammopathies where sFLC quantitation has replaced the requirement for 24h urine (Dispenzieri, *et al* 2009).

International guidelines for sFLCs are based upon the Freelite® assay (Dispenzieri, *et al* 2009, Durie, *et al* 2006). It is important to recognise that in practice different countries, or indeed laboratories may employ their own thresholds in line with normal ranges generated locally. Incorporation of new technologies such as Seralite® into clinical practice requires similar evaluation as Freelite®. This is particularly important because although different sFLC tests may give very comparable quantitation of normal polyclonal FLC, the anti-FLC antibodies in the tests may have differing affinities for an individual patients monoclonal FLC and thus give different quantitation (Campbell, *et al* 2017, Jacobs, *et al* 2014, Te Velthuis, *et al* 2016). Median Freelite® sFLC levels at diagnosis were roughly 5-fold higher than the sFLC levels measured by Seralite®. Overestimation of involved FLC levels on Freelite®, as well as another nephelometric assay (N Latex, Siemens), has been reported and discussed previously (de Kat Angelino, *et al* 2010, Tate, *et al* 2012, VanDuijn, *et al* 2015). Despite differences in absolute FLC levels between methods, a high level of diagnostic concordance was demonstrated; 99% in all LCO patients tested. Using ROC analyse in NS patients, we identified for Seralite® a dFLC level of 20mg/L to be equivalent to the GLT threshold of measurable disease on Freelite® (iFLC 100mg/L) (Durie, *et al* 2006). Using a dFLC of 20 mg/L on Seralite®, 97% of all LCO patients were considered appropriate for disease monitoring with Seralite®, compared to 98% with the 100 mg/L criterion on Freelite®.

Serum FLC testing has previously been shown to aid in the diagnosis of patients IFE negative on both serum and urine (Drayson, *et al* 2001). We studied 60 NS patients at diagnosis and in 31 found an abnormal sFLC ratio by at least one of the two sFLC methods. Overall, 23/60 (38%)

patients tested by Freelite® had an abnormal ratio and iFLC > 100mg/L and 14/35 (40%) patients tested by Seralite® had an abnormal ratio and dFLC > 20mg/L. This supports evidence for the utility of sFLC testing in patients who are negative in urine and serum IFE at diagnosis, with over a third of patients also suitable for monitoring using sFLCs. In those NS patients with elevated FLCs and abnormal ratios, the frequency of κ vs λ myelomas was 7:1 in contrast to LCO patients, where a ratio of 1.5: 1 for κ to λ at diagnosis occurred. It has been hypothesised that the higher occurrence of κ iFLC in NS myeloma may be due to variable polymerisation of light chains in λ patients, resulting in negative electrophoretic gels (Drayson, *et al* 2001, Pratt 2008), and subsequent classification of NS rather than LCO myeloma. However, in our patients the urine was negative for FLC by both IFE and immunochemical quantitation on the Luminex assay.

Follow-up of these patients revealed significant percentage reductions in sFLCs in response to therapy using both methods, supporting the use of 20 mg/L dFLC on Seralite® and reaffirming the threshold of 100 mg/L of Freelite® at presentation. Longitudinal data also confirmed the high efficacy of treatments administered to LCO patients in these recent UK clinical trials. When comparing response for LCO patients who had data for both methods, there was no difference between percentage reductions in dFLC on Seralite® vs Freelite®, despite differences in absolute dFLC levels at this time point. There were minor differences in response categorisations between these two methods within the LCO (but not OS) cohort, with more patients obtaining a CR when κ : λ ratios were determined by Seralite® compared to Freelite®. However, at the decision point in determining a good response (\leq VGPR), the methods provided the same clinical information.

We demonstrated that response by sFLC assessment is associated with survival outcomes. Patients who achieved a VGPR or CR had significantly better PFS and OS. Although stronger relationships with survival were found in the larger Freelite® cohort, consistent findings were observed for Seralite®. Patients with \geq VGPR had >60% and 46% reduced risk of death or disease progression compared to those who did not achieve a VGPR, measured by Freelite® and Seralite®, respectively. These results indicate that response to therapy assessed by sFLCs, using either assay, is prognostic for survival.

In the present study; 24h urine was not available and urine electrophoresis and IFE (as recommended for monitoring by IMWG uniform response criteria), were not included as comparison with sFLC quantitation at follow-up. Evidence indicates that measuring changes in FLC levels in serum provides a more sensitive indicator of disease activity than changes in urine (Bradwell, *et al* 2003, Dejoie, *et al* 2016a, Dejoie, *et al* 2016b, Drayson, *et al* 2001) and the addition of normalisation of sFLC levels for a stringent CR in current guidelines adds that important improved sensitivity for LCO patients (Dispenzieri, *et al* 2009, Durie, *et al* 2006, Kumar, *et al* 2016, Rajkumar, *et al* 2011). Importantly, a recent study demonstrates that response evaluated by sFLC measurements had greater prognostic significance in LCO patients than urine IFE and electrophoresis (Dejoie, *et al* 2016b). Taken together, this recent evidence and the present study confirm the utility of sFLC measurement for patient monitoring in LCO myeloma and support its application in clinical practice.

We employed the novel strategy of using the normalisation of the dFLC to classify CRs, which is in accord with the other response categories that use percentage change in dFLC. It is important to note that treatment often results in immunosuppression of the uninvolved FLC to levels below the sensitivity of the assays making the FLC ratio unreliable. Also abnormal sFLC ratios often arise in association with oligoclonal plasma cell reconstitution after high-dose melphalan. We found that the normalisation of the dFLC was associated with better PFS (measured by both assays) and OS (when measured using Seralite®). While patients were less likely to die/progress when the dFLC normalised at max response, the $\kappa:\lambda$ ratio did not provide consistent prognostic information. A significant difference was found only for PFS between a normalised vs abnormal ratio by Seralite®. This is in contrast to Dejoie *et al* where normalisation of the $\kappa:\lambda$ ratio on Freelite® predicted survival outcomes in LCO patients both post-induction and post-consolidation therapy (Dejoie, *et al* 2016b). Whether the normalisation of dFLC can provide enhanced prognostic value beyond normalisation of the $\kappa:\lambda$ ratio requires ratification in other clinical datasets.

At relapse an absolute increase in dFLC of > 30 mg/L by Seralite® was observed to be equivalent to an absolute increase in iFLC of > 200 mg/L on Freelite® and provided complete concordance on identification of progressive disease. Again, despite lower levels of absolute FLCs on Seralite® at

this time point, an increase in disease activity from remission could clearly be demonstrated.

Increases in the iFLC on Freelite® and dFLC on Seralite® should be taken into consideration with other clinical information to inform patient management. These thresholds do not necessarily imply treatment intervention but may be appropriate signals for increasing the frequency of clinical appointments and monitoring. Further, the thresholds obtained for Seralite® require confirmation in additional investigations of consecutive patient samples.

Early diagnosis and treatment intervention in myeloma is particularly important in the presence of common myeloma associated complications, such as acute kidney injury. In these cases, immediate identification of monoclonal FLCs is essential to permit prompt initiation of treatment and enable renal recovery (Hutchison, *et al* 2011a, Hutchison, *et al* 2011b). sFLC nephelometric and turbidimetric laboratory assays can return quicker results than other methods of assessing monoclonal FLCs (protein electrophoresis, immunofixation electrophoresis). However, samples are run on these analysers in large batches, and not usually on a daily basis. This route of sample shipment, testing and processing by centralised/independent laboratories can lead to delays of days to weeks in receiving patient results. Seralite® could be used by any hospital that operates a 24h laboratory service to obtain results in 10 minutes and dramatically speed up processing of urgent patient samples. In addition, the capacity to test on-site has the potential to support sFLC testing in a clinic setting. This test could be used as a tool for physicians to identify efficacy of anti-myeloma therapy and screen for relapse, and possibly provide immediate results to patients in out-patient clinics. Seralite® may be particularly useful in allowing on-site testing in clinical services who do not manage a large number of myeloma patients/only have small batches of samples. The use of Seralite® in these proposed settings needs to be tested and validated in future prospective studies.

This is the largest described study of NS and LCO myeloma patients from diagnosis to maximum response and disease progression. Both Freelite® and Seralite® were able to diagnose and monitor LCO and OS. These methods showed differences in absolute FLC levels but demonstrated good clinical concordance at diagnosis and during patient monitoring. Thus despite variations in sFLC quantitation they can provide comparable information regarding disease activity.

As a portable rapid test, Seralite® may be able to overcome delays associated with laboratory analysers to accelerate patient diagnosis and quickly inform on patient responses to therapy. Response by sFLC assessment is associated with survival outcomes in LCO myeloma. Serum FLC testing can provide sensitive monitoring for patients and should be further integrated into routine clinical practice.

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Author Contributions

JAC, GM, GJ and WG were the chief investigators on myeloma IX and XI trials. MD, JH, JC, DC, AG and JB gathered, reviewed and interpreted the data. MS was involved in the development of Seralite®. MD, JH and JC wrote the manuscript. All authors approved the manuscript.

Conflicts of interest statement

MD and JC own shares in Abingdon Health Ltd. MS is an employee of Abingdon Health Ltd. MD has an advisory role with Abingdon Health Ltd. Seralite® development was funded by Abingdon Health Ltd. JH receives research funding from Abingdon Health Ltd.

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Table I. Serum FLC parameters and response criteria for light chain only and oligosecretory patients determined by Freelite® and Seralite® at max response

	Light chain only n = 132		Oligosecretory n = 31	
Median (range)	Freelite®	Seralite®	Freelite®	Seralite®
dFLC (mg/L) at presentation	3207.8* (205–28587)	657.5 (26.6–29596)	758.8* (134.2–17670)	106.2 9 (22.4–3308)
dFLC (mg/L) at max response	19.7* (-30.0–3485.7)	7.6 (-37.6–2251.5)	3.5* (-8.6–1315.4)	2.5 (-46.5–155.8)
% reduction dFLC from presentation	99.3 (8.6–103.2)	98.7 (43.0–108.2)	99.7 (51.2–101.5)	98.3 (46.9–222.4)
Response criteria n (%)				
CR: normalised ratio	55 (41.7)	69 (52.3)	15 (48.4)	17 (54.8)
CR: normalised dFLC	57 (43.2)	73 (55.3)	20 (64.5)	20 (64.5)
VGPR	59 (44.7)	37 (28.0)	6 (19.4)	3 (9.7)
PR	14 (10.6)	21 (15.9)	5 (16.1)	5 (16.1)
SD	2 (1.5)	1 (0.8)	–	3 (9.7)

CR: complete response; VGPR: very good partial response; PR: partial response; SD: stable disease
CR by normalised $\kappa:\lambda$ ratio: 0.26–1.65 on Freelite®; 0.5–2.5 on Seralite®; CR by normalised dFLC: 0–9.85 on Freelite®; 0.02–11.6 on Seralite®. In patients who did not achieve a normal dFLC, responses were categorised as VGPR: $\geq 90\%$ reduction in serum dFLC; PR $\geq 50\%$ reduction in serum dFLC; SD $< 50\%$ reduction in serum dFLC.

*significantly higher than Seralite®, $p < .01$ (comparisons made within patient subgroup).

For the light chain only patients, there was a significant difference in response criteria between the two methods, $X^2 = 8.74$, $p < .05$. For both methods, there was a significant difference in therapy responses between LCO and OS patients ($X^2 = 7.59$, $p < .05$ Freelite®; $X^2 = 11.96$, $p < .01$ Seralite®)

Table II. Serum FLC parameters for all patients at relapse analysed using Freelite® and Seralite® Light chain only patients (n = 34) were combined with oligosecretory patients (n = 6) at relapse

All patients (n = 40)		
Median (range)	Freelite®	Seralite®
dFLC (mg/L) at max response	40.1* (0.13–1155.2)	13.7 (0–361.3)
dFLC (mg/L) at relapse	558* (227.3–5589.4)	101.2 (29.7–3993.4)
% increase dFLC from max response	1063.3* (19.8–417468)	725.8 (38.1–15810)
% normal ratio at max response	28%	30%
% abnormal ratio at relapse	100%	100%

dFLC: free light chain difference (involved FLC – uninvolved FLC)

*significantly higher than Seralite®, $p < .01$

Fig 1. Schematic of study patients characterised by type of myeloma at disease presentation

Subsets of patients with no detectable light chains in urine (non-secretory (NS) or low levels of urinary light chains (light chain only (LCO) patients not sufficient for monitoring via urine FLCs were able to be re-classified as oligosecretory patients based on an abnormal serum $\kappa:\lambda$ FLC ratio and levels sufficient to measure response to therapy.

Patients were classified according to the following guideline thresholds (GLTs) deemed to be measurable disease: serum FLC ≥ 100 mg/L and urine FLC ≥ 200 mg/g creatinine. Data is shown for all LCO patients (n = 576), which are then subdivided into patients whom had matched Seralite® and Freelite® serum FLC data. Only LCO patients with urine FLC \geq GLT or subsequently classified as OS were taken forward for further analyses (n = 3 had unmeasurable disease by both urine and serum FLCs).

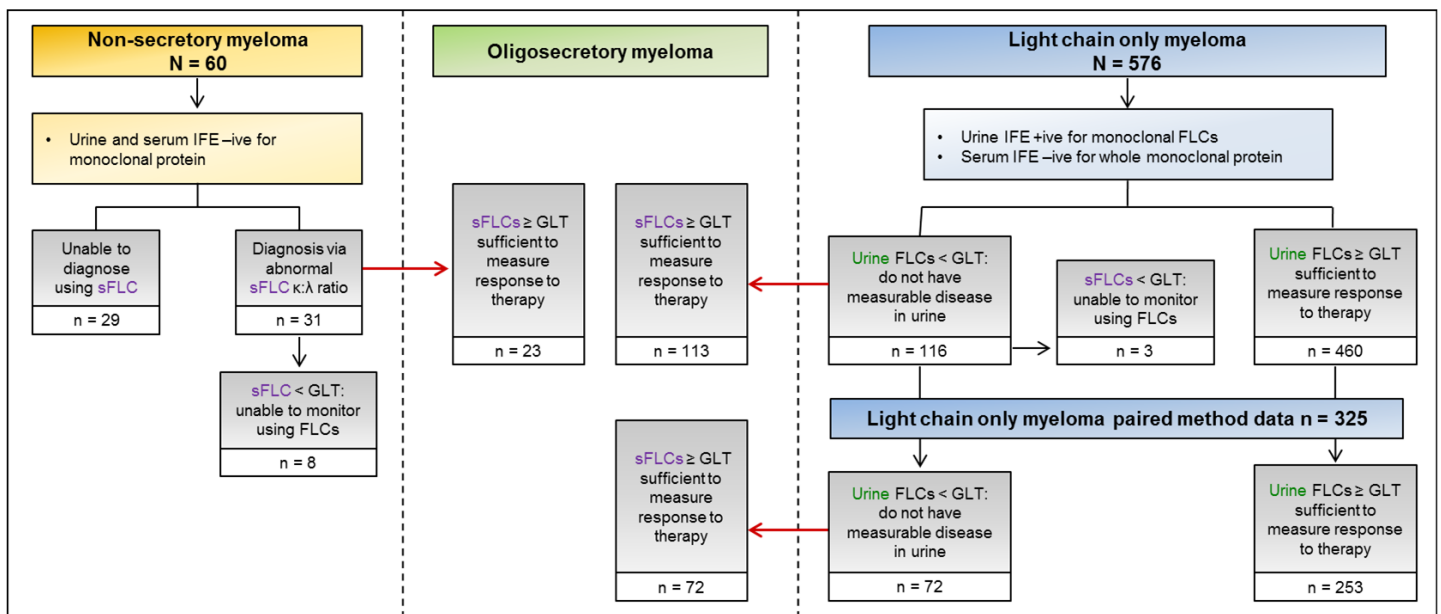


Fig 2. Involved serum free light chain levels in light chain only and oligosecretory myeloma patients at disease presentation compared between Freelite® and Seralite®

Data is shown for light chain only patients (left) and oligosecretory patients (right). Data is presented for all patients using Freelite® (n = 573; LCO = 460; OS = 113); A sub-cohort of 325 patients (LCO = 253; OS = 72) were also analysed using Seralite® and matching Freelite® data is shown separately for these specific patients. Paired data was compared between the two methods with * indicating a significant difference between Seralite® and Freelite®, $p < .001$ for both comparisons. Boxes represent the 25–75th percentile, with the line indicating the median, and whiskers represent the range

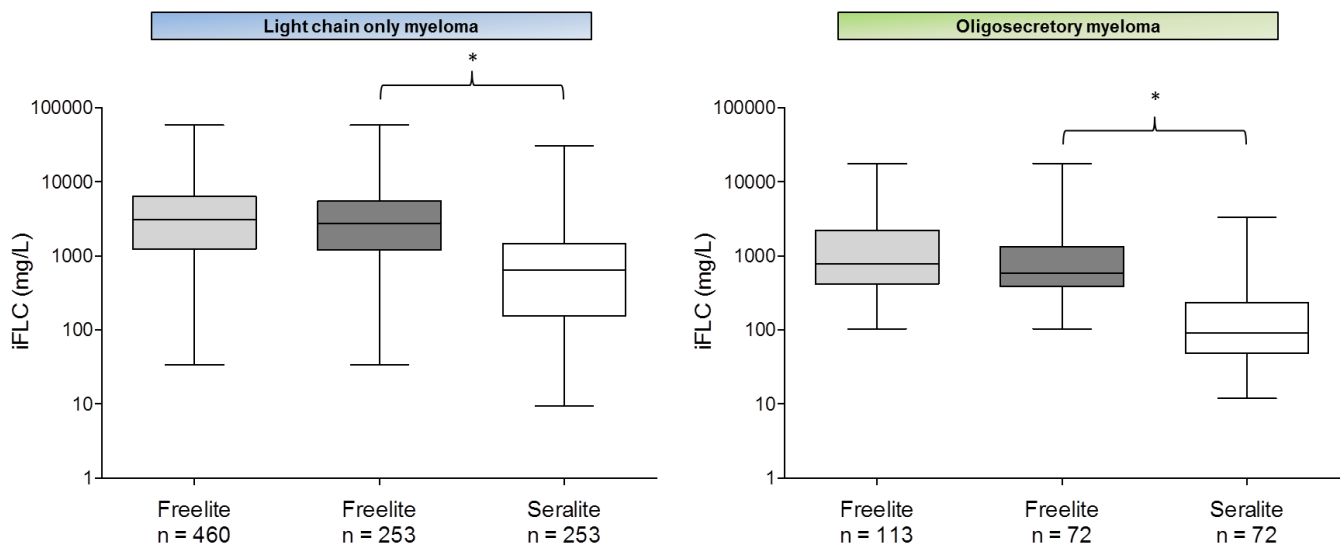


Fig 3. Comparison of the $\kappa:\lambda$ FLC ratio between Freelite® and Seralite® in serum samples from light chain only myeloma and oligosecretory patients at disease presentation

Data is presented for light chain only (LCO) patients (253; κ = 155; λ = 98) and oligosecretory (OS) patients (n = 72; κ = 40; λ = 32)

Lines represent the reference ranges for each assay: 0.26–1.65 for Freelite® and 0.5–2.5 for Seralite®. Both Freelite® and Seralite® demonstrated abnormal $\kappa:\lambda$ ratios for all LCO patients. Three OS patients showed a normal $\kappa:\lambda$ ratio by Seralite®; in these cases no monoclonal FLCs were detectable in serum by immunofixation electrophoresis

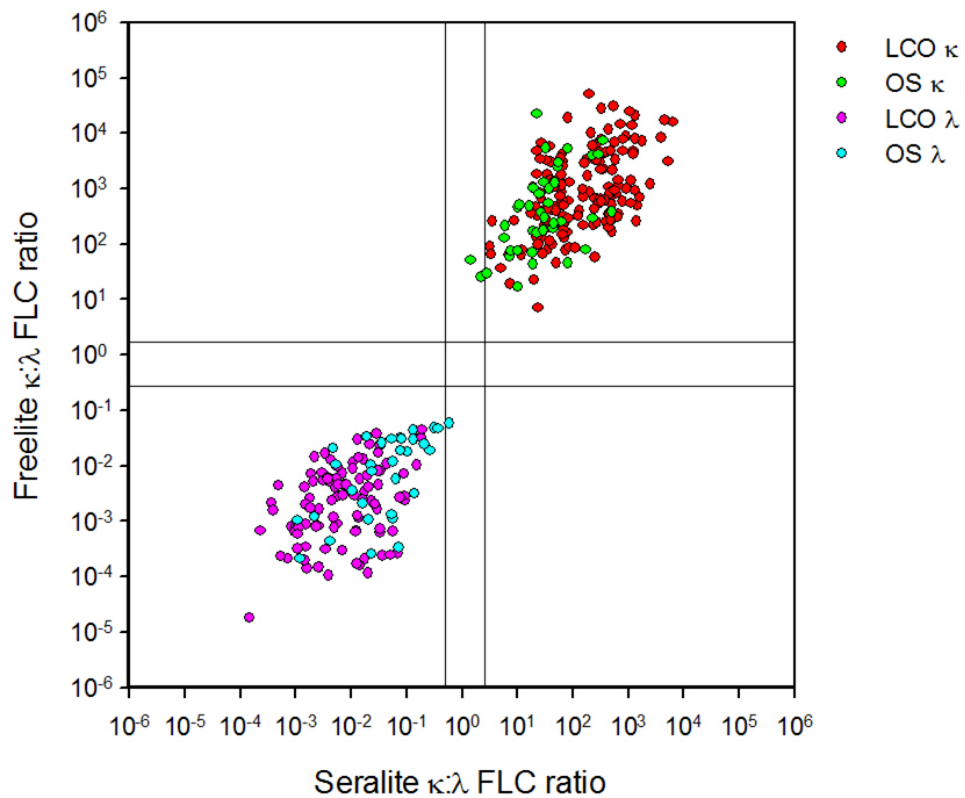


Fig 4. Survival outcomes in relation to serum free light chain response assessed by Freelite®

Progression free survival (A) and overall survival (B) according to \geq VGPR (n = 293) vs < VGPR (n = 109). Progression free survival (C) and overall survival (D) according to normalisation of the free light chain difference (dFLC) vs non-normalisation of the dFLC in patients who achieved \geq VGPR

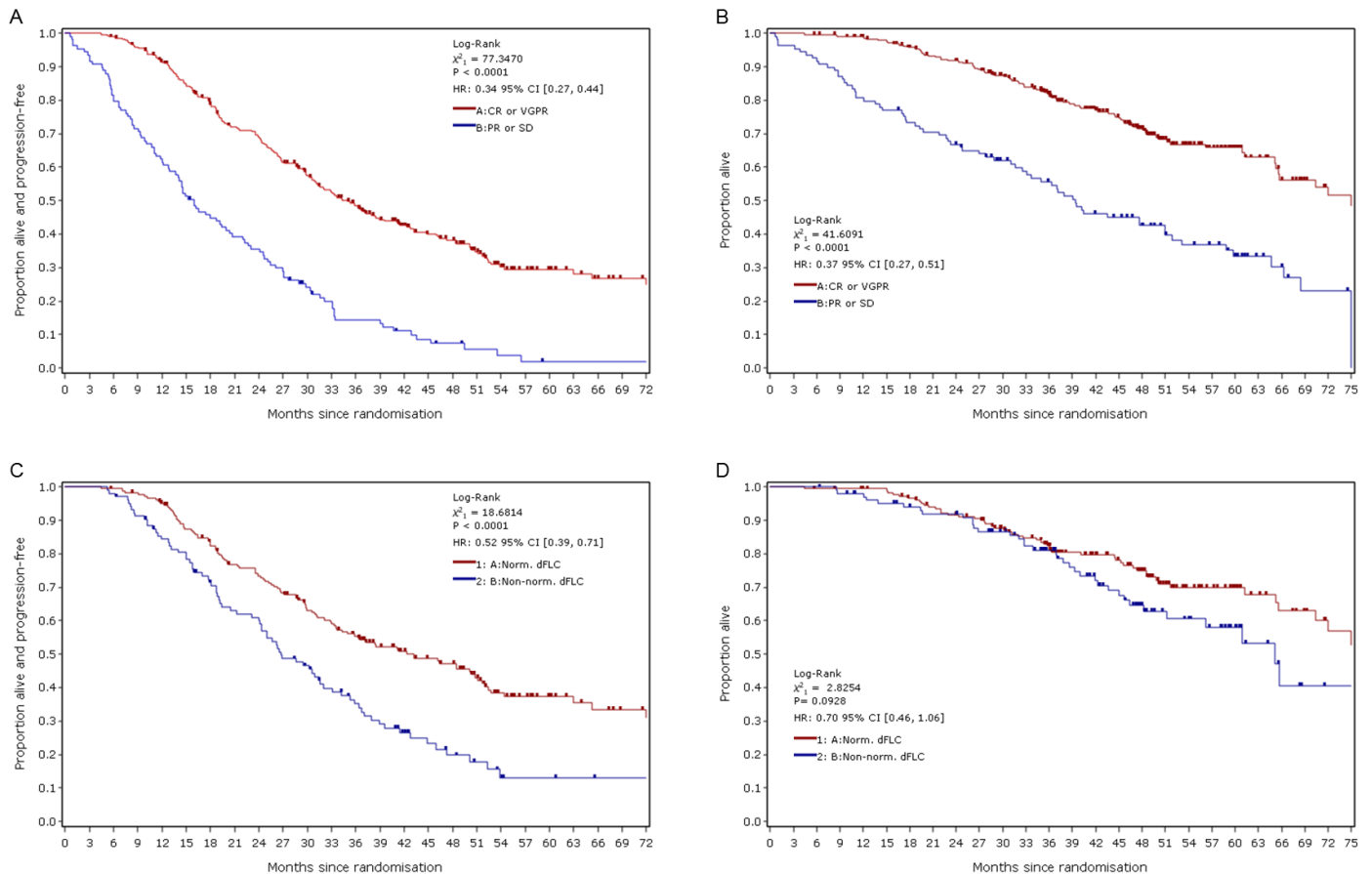
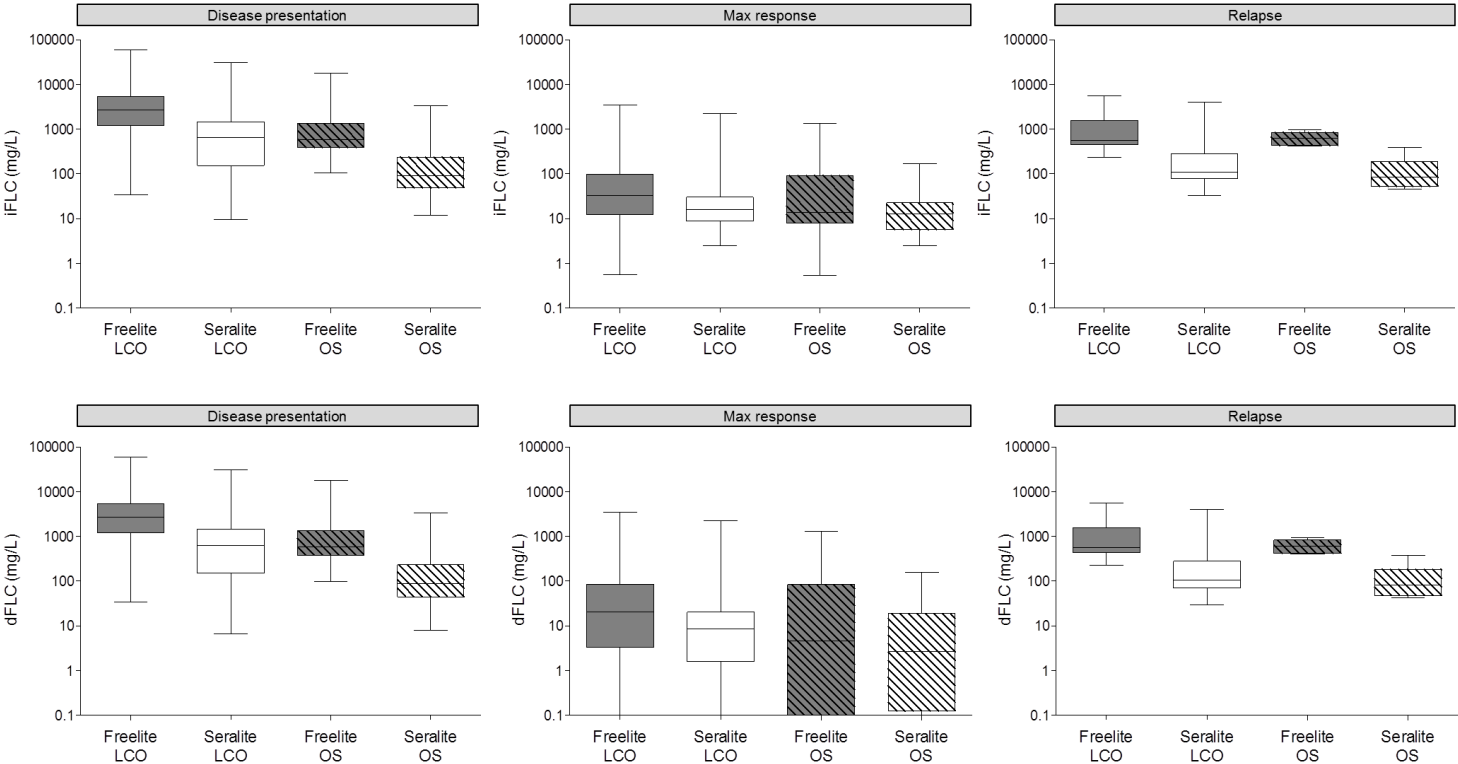


Fig 5. Longitudinal tracking of free light chain parameters measured using Freelite® and Seralite®

The involved free light chain (iFLC, top) and free light chain difference (dFLC, bottom) values are displayed for light chain only and oligosecretory myeloma patients followed from disease presentation, through to max response and subsequent relapse measured by both Freelite® and Seralite®. Patients included post-diagnosis are individuals who presented with sFLC > guideline threshold (both iFLC > 100 mg/L on Freelite® and dFLC > 20 mg/L on Seralite®). Boxes represent the 25–75th percentile, with the line indicating the median, and whiskers represent the range



Supplementary Table I. Serum free light chain parameters at disease presentation in light chain only and oligosecretory myeloma compared between measurement techniques

All patients serum was analysed using Freelite® (n = 573); 325 patients were also analysed using Seralite® and matching Freelite® data is shown separately for these specific patients

	Light chain only			Oligosecretory		
	Freelite®	Freelite®	Seralite®	Freelite®	Freelite®	Seralite®
Median (range)	n = 460 (κ = 274; λ = 186)	n = 253 (κ = 155; λ = 98)		n = 113 (κ = 66; λ = 47)	n = 72 (κ = 40; λ = 32)	
iFLC (mg/L)	3091.0 (33.8–58246)	4408.4 (33.8–38246)	638.0 (9.4–30480)	780.0 (103.4–17674)	1744.9 (103.4–17760)	91.2 (11.9–3312)
k:λ ratio κ patients	812.1 (2.31–265625)	3127.5 (7.0–51037)	152.6 (3.2–6288)	383.1 (9.6–7901.6)	1616.5 (16.9–22540)	27.8 (1.4–500.8)
κ:λ ratio λ patients	0.002 (0–0.117)	0.005 (0–0.045)	0.006 (0–0.186)	0.010 (0–0.48)	0.017 (0–0.059)	0.055 (0.001–0.583)

iFLC: involved free light chain. For both methods, patients with LCO myeloma had significantly higher serum iFLC compared with oligosecretory myeloma ($p < .001$).

Normal ratio reference ranges for each assay: 0.26–1.65 Freelite®; 0.5–2.5 Seralite®

Supplementary Table II. Serum free light chain parameters at disease presentation in nonsecretory myeloma patients analysed using Freelite® and Seralite®

N = 60		Freelite®				Seralite®			
	Patient	κ (mg/L)	λ (mg/L)	Ratio	dFLC (mg/L)	κ (mg/L)	λ (mg/L)	Ratio	dFLC (mg/L)
Elevated κ & increased ratio n = 27	1	25.8	6.2	4.2	19.6	5.7	2.6	2.2	3.1
	2	26.7	20.7	1.3	6	61.4	14.4	4.3	47
	3	73.2	10.7	6.8	62.4	12.2	10.3	1.2	1.9
	4	31.2	1.3	24.0	29.9	15.3	2.5	6.1	12.8
	5	33.1	6.9	4.8	26.2	17.8	2.5	7.1	15.3
	6	83.3	3.5	23.9	79.8	22.6	2.5	9.0	20.1
	7	86.9	14.5	6.0	72.4	11.2	17.9	0.6	6.7
	8	87.7	9.6	9.2	78.2	15.8	9	1.8	6.8
	9	143.2	2	71.6	141.2				
	10	160.3	9.5	16.9	150.8				
	11	195.5	11	17.8	184.5	54.9	16	3.4	38.9
	12	297.5	10.3	28.8	287.2	20.2	5	4.0	15.2
	13	302.2	8.7	34.9	293.5	51	2.6	19.6	48.4
	14	304.1	9.6	31.7	294.5	46.6	2.5	18.6	44.1
	15	408	0.6	680	407.4	24.8	2.5	9.9	22.3
	16	418.8	9	46.6	409.8	736	13.2	55.8	722.8
	17	427.4	0.7	602	426.7	24.8	2.5	9.9	22.3
	18	451.8	8.3	54.6	443.6	60.8	2.5	24.3	58.3
	19	458.1	13.4	34.1	444.7	26.6	21.6	1.2	5.0
	20	487.6	3	163.6	484.6				
	21	509	2.8	181.8	506.2				
	22	564	8.6	65.7	555.4	66.2	11.8	5.6	54.4
	23	577	7.8	74.0	569.2	40.1	2.5	16.0	37.6
	24	823	1.4	579.6	821.6				
	25	1127	0.5	2254	1126.5				
	26	1192	1.2	1010.2	1190.8				
	27	1758	1.3	1362.8	1756.7	81.8	2.5	32.7	79.3
Elevated λ & decreased ratio n = 4	28	7.98	164.21	0.050	156.23				
	29	4.87	255.06	0.019	250.19	12.00	356.00	0.034	344.00
	30	4.95	639.86	0.008	634.91	5.70	534.00	0.011	528.30
	31	7.7	1997	0.004	1989.3				
FLC suppression Unreliable ratio n = 18	32	0.4	11.2	0.03	10.8	4.7	15.7	0.3	11
	33	0.7	1.4	0.5	0.8	2.5	2.5	1.0	0
	34	0.9	1.9	0.5	1				
	35	4.3	9.2	0.5	4.9				
	36	5.1	8	0.6	2.9	5.5	3	1.8	2.5
	37	0.7	1.2	0.6	0.5	2.8	2.5	1.1	0.3
	38	0.8	1.1	0.8	0.3	6.9	4.3	1.6	2.6
	39	5.2	6.8	0.8	1.6	4.9	4.8	1.0	0.1
	40	10	10	1.0	0				
	41	7.9	7.3	1.1	0.6	2.5	2.5	1.0	0
	42	9.5	8.2	1.2	1.3	5.3	3.5	1.5	1.8
	43	0.7	0.5	1.4	0.2	2.5	2.5	1.0	0
	44	1.3	0.9	1.4	0.4				
	45	8.4	5.8	1.5	2.6	10	9.4	1.1	0.6
	46	6.1	2.8	2.2	3.3				
	47	3.7	1.6	2.4	2.1				
	48	8.5	2.2	3.8	6.2	5.5	2.5	2.2	3
	49	4.8	1.3	3.8	3.5	2.5	2.5	1.0	0
K or λ normal/ borderline or normal ratio n = 11	50	3.8	25.1	0.2	21.3				
	51	7.06	24.74	0.3	17.68				
	52	9.2	36.8	0.3	27.6				
	53	8.8	13.1	0.7	4.4				
	54	40.3	51.6	0.8	11.3				

55	35.6	37.4	1.0	1.8	27.6	33.4	0.8	5.8
56	13.5	12.6	1.1	0.9				
57	27.1	21.4	1.3	5.8				
58	25.7	17.3	1.5	8.4	24.2	17.5	1.4	6.7
59	13.5	9.2	1.5	4.3				
60	22.6	14.1	1.6	8.5	16.7	26.9	0.6	10.2

Normal ratio reference ranges for each assay: 0.26–1.65 Freelite®; 0.5–2.5 Seralite®

Grey boxes indicate disagreement between Freelite® and Seralite® for patients with elevated FLCs, where one method indicates a perturbed ratio and the other presents as normal.

dFLC; free light chain difference (involved FLC – uninvolved FLC): in cases with suppressed or normal FLC the larger FLC value was taken to be the involved.